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Novel Antioxidant Properties of Ghrelin and Oleuropein Versus Lipopolysaccharide-Mediated Renal Failure in Rats

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Abstract Ghrelin and oleuropein have recently been shown to bear beneficial effects against oxidative stress in our reports. The aim of this study was to evaluate the antioxidant abilities of ghrelin and oleuropein in a lipopolysaccharide (LPS)-induced nephrotoxicity model. The Sprague-Dawley male rats were divided into LPS, the ghrelin$_1$ plus LPS (Ghr$_1$+LPS), the ghrelin$_4$ plus LPS (Ghr$_4$+LPS), the oleuropein$_{10}$ plus LPS (Ole$_{10}$+LPS) and the oleuropein$_{15}$ plus LPS (Ole$_{15}$+LPS) groups. 1 or 4 nmol/rat of ghrelin as subcutaneous and oleuropein 10 or 15 mg/kg as oral were administrated to ghrelin and oleuropein pretreated rats and vehicle injected to LPS group for ten consecutive days. All of the groups received LPS (once; 5 mg/kg) in the 11th day of the treatment as intraperitoneally. LPS-induced nephrotoxicity was manifested by a significant elevation in renal function tests (BUN and creatinine; $P < 0.001$) and histopathology findings in LPS group in comparison with the ghrelin and oleuropein pretreated rats. Renal lipid peroxidation was significantly higher in the LPS-treated animals than the ghrelin and oleuropein pretreated groups ($P < 0.05$). In contrast, glutathione peroxidase (GPx), catalase and superoxide dismutase activities were significantly higher in Ghr$_4$+LPS-treated rats than LPS group ($P < 0.05$). GPx activity was also significantly higher in Ole$_{15}$+LPS-treated rats than LPS group ($P < 0.05$). Regarding nitrosative stress, renal nitrite content was significantly lower in Ghr$_4$+LPS and Ole$_{15}$+LPS groups than LPS group ($P < 0.05$). These results suggest that ghrelin and oleuropein have beneficial antioxidant properties versus LPS-induced renal failure in rats.

Keywords Ghrelin · Oleuropein · Lipopolysaccharide · Nephrotoxicity · Oxidative stress

Introduction

Lipopolysaccharide (LPS)-induced nephrotoxicity is a well accepted model for renal failure in rats (Doi et al. 2009). Endotoxin, a component of the outer membrane of Gram-negative bacteria, is involved in the pathogenesis of sepsis, and LPS injection has been widely applied for sepsis research (Remick et al. 2000; Doi et al. 2009). LPS produces reactive oxygen species (ROS) and nitric oxide (NO) in macrophages. These molecules are involved in oxidative and nitrosative stresses, inflammation and endotoxic shock (Kim et al. 2004). The consequence of oxidative and nitrosative stresses are multiple and invariably ominous (Noiri et al. 1996). The excessive production of ROS and NO in the human body is involved in the pathogenesis of various diseases including atherosclerosis, diabetes mellitus and inflammatory diseases such as chronic renal failure and end-stage renal disease (ESRD) (Obay et al. 2008; Laviano et al. 2010; Neamati et al. 2011). ROS and NO, collectively contribute to the loss of cell viability either via necrotic or apoptotic pathways (Bonfoco et al. 1995; Noiri et al. 1996). ROS, such as superoxide anion, hydrogen

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peroxide and hydroxyl radical, which are generated as byproducts of oxidative metabolism in mitochondria, can interact with biomolecules such as DNA, RNA, protein, and lipids subsequently damaging various cellular components (Kheradmand et al. 2010; Neamati et al. 2011). NO acts as an intracellular messenger and regulates cellular functions such as vasorelaxation and inflammation. NO also has an important role in the elimination of pathogens and tumor cells (Kim et al. 2004). The physiological and pathophysiological implications of NO depend on its local concentration, the type of nitric oxide synthase (NOS) isozyme involved in NO generation and substrate availability (Korhonen et al. 2005; Sun et al. 2006; Slomiany and Slomiany 2010). Overproduced NO is oxidized to reactive nitrogen species and results in the disruption of cell signaling and uncontrolled systemic inflammation and septic shock (Kim et al. 2004). Moreover, a high level of NO generated by inducible NOS (iNOS) in response to proinflammatory cytokines and bacterial LPS, is associated with the induction of apoptosis (Chanvorachote et al. 2005; Korhonen et al. 2005; Maa et al. 2008; Slomiany and Slomiany 2010). In fact, binding of LPS to Toll like receptor launches intracellular signaling, which produces activation of nuclear factor-kappa B (NF-κB) and subsequent expression of iNOS (Amiraslani et al. 2012). In addition, the reaction between superoxide ion and NO resulting in an almost instantaneous generation of peroxynitrite which induces acute renal ischemia and contributes to the pathophysiology of renal failure (Noiri et al. 1996, 2001).

Ghrelin has been identified as an endogenous ligand for growth hormone secretagogue receptor (GHSR) that regulates growth hormone (GH) secretion, regulates food intake, increases appetite and contributes to insulin release and energy homeostasis (Kojima and Kangawa 2005; Obay et al. 2008; Kheradmand et al. 2010, 2011; Neamati et al. 2011). It has been detected in a large number of tissues and cell types, including hypothalamus, small intestine, pancreas, placenta, pituitary, brain, lung, and kidney (Fernandez-Fernandez et al. 2005; Ghelardoni et al. 2006; Takeda et al. 2006; Neamati et al. 2011). It seems, the renal protective effects of ghrelin be due to induction of constitutive NOS (cNOS) and cyclic guanosine mono phosphate (cGMP) in the kidney by GH and insulin growth factor 1 (Takeda et al. 2006; Neamati et al. 2011). We know, renal failure is a state of GH resistance, and based on our previous report, it is a state of ghrelin resistance too (Neamati et al. 2011). Therefore, one potential treatment for cachexia in renal failure is the use of the orexigenic hormone ghrelin as exogenous injection (Nakazato et al. 2001; Sun et al. 2004; DeBoer et al. 2008). Although ghrelin levels are already elevated above normal in cachexia-associated disease states such as cancer, heart and renal failures, administration of supra physiological doses of ghrelin has been shown to increase food intake in human subjects with renal failure (Hataya et al. 2003; Ayala et al. 2004; Nagaya et al. 2004; Neary et al. 2004; DeBoer et al. 2008). Ghrelin also indicated an antioxidant role in the kidney of rats upon our previous report (Neamati et al. 2011). We therefore hypothesized that exogenous ghrelin may have a potential effect against endotoxin-mediated renal failure.

Despite the wide body of evidence linking the orexigenic properties of ghrelin in patients with renal failure, the present study has attempt to clarify the antioxidant properties of ghrelin versus LPS-induced renal failure. For this purpose, we used oleuropein a phenolic compound in olive leave extract, as an antioxidant agent based on our previous reports (Alirezaei et al. 2011, 2012a, b, 2014), to investigate beneficial effects of the hormone. In this regard, it has been shown that oleuropein is rapidly absorbed from the intestine with t_{max} of 2 h reaching a peak of 200 ng/ml of plasma after administration of 15–20 mg/kg oleuropein in rats (Alirezaei et al. 2012b). Hence, the high availability of oleuropein in its active form in vivo may explain the positive impact on the renal function, antioxidant enzymes and NOS in the current study.

Materials and Methods

Rat lyophilised acylated ghrelin (*n*-octanoylated research grade) was prepared from Tocris Cookson Ltd. (Bristol, UK). Oleuropein was purified from olive leaf extract according to our previous reports (Alirezaei et al. 2012a, b). LPS (*Salmonella typhosa* cell wall) was prepared from Sigma® Chemical Company (Sigma, St. Louis, MO). Thiobarbituric acid (TBA) and Griess reagent were supplied from Merck® Chemical Company (Merck, Germany). The GPx and superoxide dismutase (SOD) kits were obtained via Randox® Company (Antrim, UK) and the BUN and creatinine kits were prepared by PARS AZMOON® Company (Tehran, Iran). Other chemicals used were of analytical grade.

Experimental Design

A total of 40 adult male Sprague-Dawley rats (weighing 200 ± 20 g) were housed in temperature-controlled conditions under a 12:12-h light/dark photocycle with food and tap water supplied ad libitum. All rats were treated humanely and in compliance with the recommendations of Animal Care Committee for Lorestan University of Medical Sciences (Khorramabad, Iran) with Approval Number: SM 90/25. All of experimental procedures were carried out between 08.00 and 10.00 am for prevention of circadian

rhythm changes among days. The rats were divided into five equal groups (n = 8 rats per group) and treated daily for 10 consecutive days as follows: the LPS group received 0.25 ml physiological saline as subcutaneously at 8.00 am daily, the ghrelin$_1$ plus LPS group (Ghr$_1$+LPS) received ghrelin (1 nmol/rat as subcutaneously at 8.00 am daily), the ghrelin$_4$ plus LPS group (Ghr$_4$+LPS) received ghrelin (4 nmol/rat as subcutaneously at 8.00 am daily), the oleuropein$_{10}$ plus LPS (Ole$_{10}$+LPS) group received purified oleuropein (10 mg/kg BW orally by gavage at 8.00 am daily), and the oleuropein$_{15}$ plus LPS (Ole$_{15}$+LPS) group received purified oleuropein (15 mg/kg BW orally by gavage at 8.00 am daily). The dose of 1 nmol ghrelin/rat is similar to the physiological concentration of circulating ghrelin in the fasting state of rats (Wren et al. 2001; Fernandez-Fernandez et al. 2005), and dose of 4 nmol ghrelin/rat is near to the dose used in our recent study (Neamati et al. 2011). In this setting, it has been demonstrated that 1 h after exogenous administration of 1 nmol of ghrelin is able to induce a significant elevation (2.4- to 2.6-fold increase) in serum levels of total ghrelin (Wren et al. 2001; Fernandez-Fernandez et al. 2005). The doses of oleuropein were chosen according to our previous reports (Alirezaei et al. 2012a, b). Ghrelin and oleuropein were dissolved in sterile physiologic saline solution before administration, daily. In the 11th day of the treatment, all groups received one dose of *Salmonella typhosa* cell wall suspension (5 mg/kg BW) as intraperitoneally. The animals were administrated under conscious conditions after careful handling to avoid any stressful influence. The weight of rats was measured at the end of the experiment and there was no significant difference among the groups. One day after the LPS injection, the rats were sacrificed using light diethyl ether anesthesia (Dagenham, UK) and blood samples were collected via cardiac puncture in order to provide serum. Then both kidneys were removed and carefully cleaned of fat and adhering. The sera and right kidney samples were stored at −70 °C for later biochemical analysis and left kidneys were fixed in 10 % buffered formalin for histopathological findings.

Histopathological Assessment

The kidney samples of the experimental groups were processed routinely for paraffin embedding. Sections were cut at 5 μm thicknesses (Leica, Germany), and stained with hematoxylin and eosin. The sections were then viewed under light microscope to detect eventual histopathological changes. Hydropic degeneration of epithelium and glomerulonephritis were evaluated in cortex, which scored according to the degree of epithelial swelling and corpuscular changes in percent, using a 0.0 through 4 grading system. 0.0 = no lesion; 1 = ≤15 % (mild); 2 = 16–30 % (moderate); 3 = >30 % (severe) for tubular swelling. 0.0 = no lesion; 1 = ≤10 %; 2 = 10–15 %; 3 = >16 % for glomerular change. Interstitial nephritis was ranked as 0.0 = normal; 1 = mild; 2 = moderate; and 3 = severe. The semiquantitative evaluation of renal damage stated in the Table 1.

BUN and Creatinine Measurement

Blood urea nitrogen (BUN) and creatinine concentrations of sera were measured chemically according to the manufacturer's instructions of the kits. Serum BUN concentration was measured according to the Berthelot's method and creatinine determination based on the Jaffe's method as described previously (Noiri et al. 2001), by a spectrophotometer (S2000 UV model; WPA, Cambridge, UK). BUN and creatinine results were expressed as milligram per deciliter (mg/dl) of serum.

Tissue Preparation for Measurement of Protein, Lipid Peroxidation, Antioxidant Enzyme Activities and NO Assay

The rat kidneys were thawed and manually homogenized in cold phosphate buffer (0.1 M, pH 7.4, containing 5 mM EDTA) and debris removed by centrifugation at 2000×*g* for 10 min (Centrifuge 5415 R; Rotofix 32A, Germany). Supernatants were recovered and used for protein measurement, lipid peroxidation value, antioxidant enzyme activities, and nitrite content. Protein content of kidney supernatants was determined using a colorimetric method of Lowry with bovine serum albumin as a standard (Lowry et al. 1951).

Measurement of Lipid Peroxidation

The amount of lipid peroxidation was indicated by the content of thiobarbituric acid reactive substances (TBARS) in the kidney. Tissue TBARS determined by following production of thiobarbituric acid reactive substances as described previously (Subbarao et al. 1990). In short, 40 μl of supernatant was added to 40 μl of 0.9 % NaCl and 40 μl of deionized H_2O, resulting in a total reaction volume of 120 μl. The reaction was incubated at 37 °C for 20 min and stopped by the addition of 600 μl of cold 0.8 mol/l hydrochloride acid, containing 12.5 % trichloroacetic acid. Following the addition of 780 μl of 1 % TBA, the reaction was boiled for 20 min and cooled at 4 °C for 1 h. In order to measure the amount of TBARS produced by the homogenate, the cooled reaction was spun at 1500×*g* in a microcentrifuge for 20 min and the absorbance of the supernatant was spectrophotometrically read at 532 nm using an extinction coefficient of 1.56×10^5/mol cm. The blanks for all of the TBARS assays contained an additional 40 μl

Table 1 The effects of lipopolysaccharide (LPS), ghrelin and oleuropein on semiquantitative histopathological findings in renal tissue of the experimental groups

Lesions	LPS	Ghr_1+LPS	Ghr_4+LPS	Ole_{10}+LPS	Ole_{15}+LPS
Subcapsular swelling (%)	57.50	32.50	16.66	28.10	33.73
Subcortical swelling (%)	15.83	36.66	6.25	24.30	16.48
Total swelling (%)	36.66	34.58	11.45[a]	26.20	25.10
Glomerular changes (%)	15.68	14.47	16.66	8.65[b]	10.60[b]
Interstitial nephritis (unit)	2.16	1.50	1.16[c]	1.28	1.00[c]

All of the groups received LPS (once; 5 mg/kg) as intraperitoneally. Data were expressed as mean (n = 8 rats per group)

Ghr_1+LPS ghrelin 1 nmol/rat plus lipopolysaccharide, *Ghr_4+LPS* ghrelin 4 nmol/rat plus lipopolysaccharide, *Ole_{10}+LPS* oleuropein 10 mg/kg plus lipopolysaccharide, *Ole_{15}+LPS* oleuropein 15 mg/kg plus lipopolysaccharide

[a] Total swelling was more significant lower in Ghr_4+LPS-trated rats when compared to the other groups

[b] Both oleuropein dosages, 10 and 15 mg/kg, could decrease the effects of LPS in case of glomerular changes in comparison with ghrelin pretreated rats

[c] Interstitial nephritis was mild in Ghr_4+LPS and Ole_{15}+LPS groups in contrast to the other groups

of 0.9 % NaCl instead of homogenate as just described. TBARS results were expressed as nmol per milligram of tissue protein (nmol/mg protein).

Measurement of GPx Activity

The activity of glutathione peroxidase (GPx) was evaluated with Randox GPx detection kit according to the manufacturer's instructions. GPx catalyse the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was measured spectrophotometrically against blank at 340 nm. One unit (U) of GPx was defined as 1 μmol of oxidized NADPH per min per milligram of tissue protein. The GPx activity was expressed as unit per milligram of tissue protein (unit/mg protein).

Measurement of CAT Activity

Tissue catalase activity was assayed using the method described by Claiborne (1986). The reaction mixture (1 ml) consisted of 50 mM potassium phosphate (pH 7.0), 19 mM H_2O_2, and a 25 μl sample. The reaction was initiated by the addition of H_2O_2 and absorbance changes were measured at 240 nm (25 °C) for 30 s. The molar extinction coefficient for H_2O_2 is 43.6/M cm. The catalase (CAT) activity was expressed as the unit that is defined as μmol of H_2O_2 consumed per min per milligram of tissue protein (unit/mg protein).

Measurement of SOD Activity

The activity of SOD was evaluated with Randox SOD detection kit according to the manufacturer's instructions. The role of SOD is to accelerate the dismutation of the toxic superoxide (O_2^-) produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity is then measured by degree of inhibition of this reaction. One unit of SOD is that which causes 50 % inhibition of the rate of reduction of INT under the conditions of the assay. SOD activity was recorded at 505 nm through a standard curve and it was expressed as unit per milligram of tissue protein (unit/mg protein).

NO Assay (Nitrite Content)

The amount of total stable nitrite, the end product of NO generation, was determined by a colorimetric method, as described previously (Kim et al. 2002, 2004). In brief, 50 μl of tissue homogenate was mixed with 100 μl of Griess reagent (1 % sulfanilamide, 0.1 % naphthylethylenediamine dihydrocholoride, and 2.5 % H_3PO_4) and 1850 μl distilled water. After 10 min of incubation at room temperature, absorbance was read at 540 nm. The blank was prepared with the same method however, instead of 50 μl of the tissue homogenate, 50 μl of distilled water was applied. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve and results were expressed as millimol per milligram of tissue protein (mmol/mg protein).

Statistical Analysis

All variables were tested for normal and homogeneous variances by Leven's statistic test. All results are presented as mean ± SEM. The statistical differences were applied among the all groups by one-way analysis of variance

(ANOVA) with Tukey's post hoc analysis. Statistical analysis was performed using the statistical package GraphPad PRISM version 5 (GraphPad Software Inc., San Diego, CA, USA). The calculated P values of less than 0.001 for BUN and creatinine tests and 0.05 for the other parameters were considered statistically significant.

Results

Epithelial swelling was prominently reduced in both ghrelin and oleuropein pretreated rats, however it was more significant in Ghr_4+LPS group when compared to the other groups. Moreover, interstitial nephritis was mild in Ghr_4+LPS and Ole_{15}+LPS groups and moderate in Ole_{10}+LPS-treated rats. There was no significant difference between LPS group and Ghr_1+LPS-treated rats. Both oleuropein dosages, 10 and 15 mg/kg, could decrease glomerulonephritis induced by LPS. In contrast, in ghrelin pretreated groups there was no variable considerable change in case of glomerulonephritis (Fig. 1; Table 1).

Administration of *Salmonella typhosa* cell wall (LPS) significantly increased BUN (as a renal function test) in sera of the LPS-treated rats when compared to the other groups ($P < 0.001$). The BUN concentration was similar in pretreated rats and there was no significant difference among ghrelin-pretreated rats and oleuropein-pretreated groups ($P > 0.05$; Fig. 2).

Injection of LPS also significantly increased creatinine in sera of the LPS-treated rats when compared to the ghrelin- and oleuropein-pretreated groups ($P < 0.001$). The creatinine concentration was similar in pretreated rats and there was also no significant difference among ghrelin-pretreated rats and oleuropein-pretreated groups ($P > 0.05$; Fig. 3). Indeed, protective effect of ghrelin and oleuropein was manifested by evaluation of renal function tests, BUN and creatinine, against LPS-induced nephrotoxicity in rats.

Regarding oxidative stress, TBARS concentration (as a lipid peroxidation marker) increased significantly in LPS-treated rats when compared to the other groups ($P < 0.05$). The concentrations of TBARS in the Ghr_4+LPS group decreased significantly as compared to the Ole_{10}+LPS-treated rats ($P < 0.05$). In fact, ghrelin at dose 4 nmol/rat was able to suppress lipid peroxidation in comparison with oleuropein 10 mg/kg in rats (Fig. 4).

The mean values ± SEM of the antioxidant enzyme activities (GPx, CAT, and SOD) of the kidney homogenates are presented in Figs. 5, 6, and 7. The GPx activity in Ghr_4 plus LPS-treated rats and Ole_{15} plus LPS group was significantly higher when compared to the LPS group ($P < 0.05$). Ghrelin and oleuropein treatments at the doses of 1 nmol/rat and 10 mg/kg were indicated similar GPx activity such as LPS-treated rats ($P > 0.05$; Fig. 5).

CAT activity was significantly higher in the Ghr_4 plus LPS-treated group when compared to the LPS, Ghr_1+LPS, Ole_{10}+LPS and Ole_{15}+LPS groups ($P < 0.05$). Although the Ghr_1+LPS and Ole_{15}+LPS groups indicated slightly higher CAT activities when compared to LPS-treated rats, these enhancements were not significant ($P > 0.05$; Fig. 6).

SOD activity was significantly higher in the Ghr_4 plus LPS-treated group as compared to the LPS-treated rats ($P < 0.05$). The Ghr_1 plus LPS group and oleuropein-treated rats also indicated slightly higher SOD activity when compared to LPS group, however it was not significant ($P > 0.05$; Fig. 7).

There was a significant increase in nitrite content of the LPS-treated rats in comparison with Ghr_4+LPS and Ole_{15}+LPS groups ($P < 0.05$). There was no significant difference among LPS-treated rats with Ghr_1+LPS and Ole_{10}+LPS groups ($P > 0.05$; Fig. 8).

Discussion

In the present study, we provide experimental support for the hypothesis that ghrelin may have a preventional role in LPS-induced nephrotoxicity, in part by significantly inhibiting the production of ROS and NO, important mediators in the pathophysiology of renal failure. On the basis of previous reports and the current findings, it appears that the oxidative response of LPS in kidney includes the production of ROS, which leads to the induction of iNOS, elevation of peroxynitrite in toxic amounts subsequently, nitrosative stress (Kim et al. 2004). In this study we showed that ghrelin and oleuropein elevate renal antioxidant status and decrease lipid peroxidation. These beneficial effects of both antioxidant agents were associated with enhanced GPx, CAT and SOD activities parallel to decrease in oxidative and nitrosative processes. The present study demonstrated that chronic administration of ghrelin and oleuropein could promote antioxidant enzyme activities and decreased NO (as shown by nitrite content) in rat kidneys particularly in the doses 4 nmol of ghrelin and 15 mg of oleuropein.

As previously mentioned, we have studied the use of both ghrelin and oleuropein to evaluate antioxidant abilities of the ghrelin in a LPS-induced renal failure. Renal failure is a state of growth hormone (GH) resistance and based on the available data it is a state of ghrelin resistance too. In this regard, exogenous administration of the hormone may overcome ghrelin resistance at target organs, improve metabolic alterations, and result in clinical benefit (Laviano et al. 2010; Neamati et al. 2011). In the present study, ghrelin treatment demonstrated to attenuate toxic effects of ROS and NO possibly via increasing renal blood flow and upregulation of renal GHSR during sepsis in rats (Wang

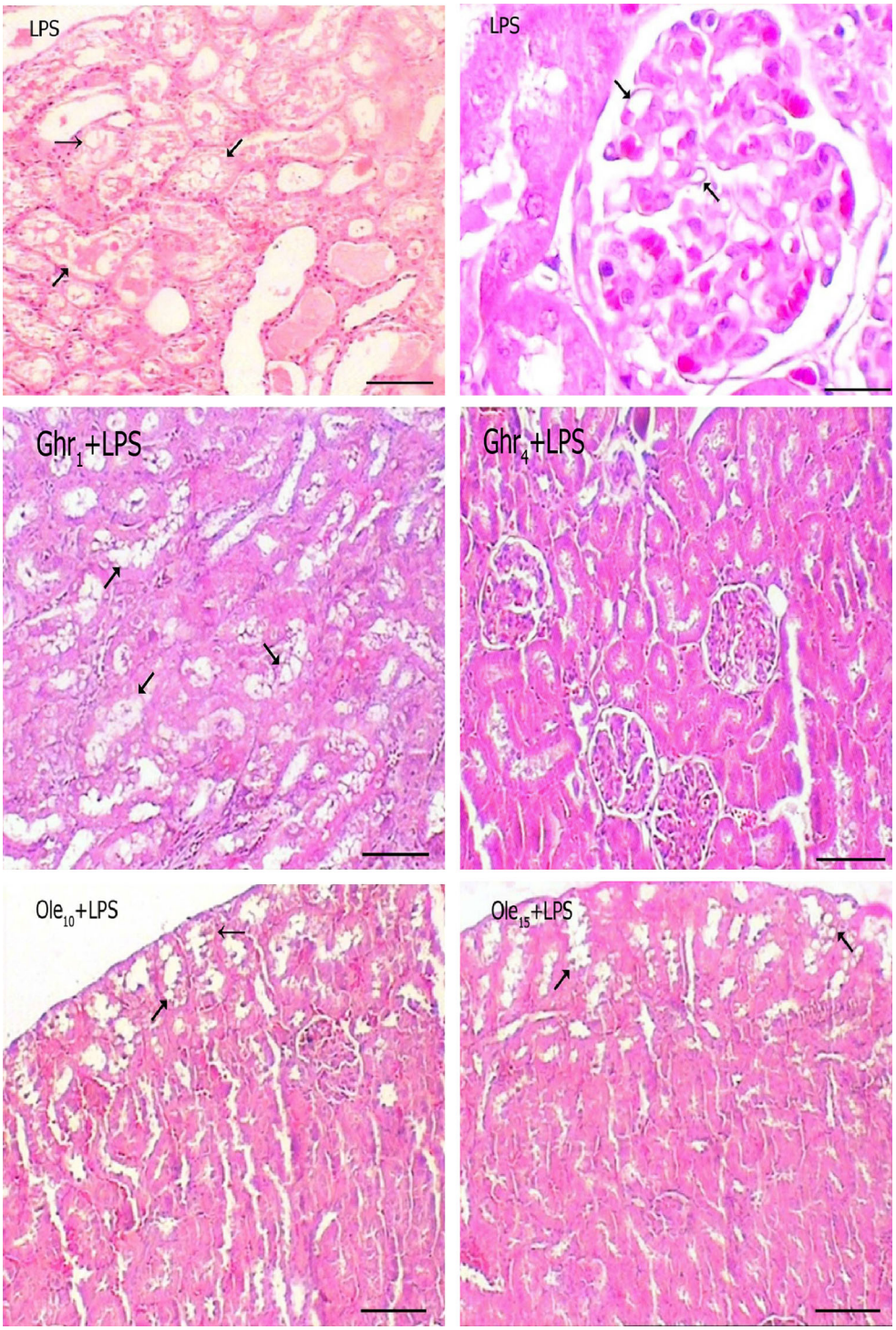

Fig. 1 The effects of lipopolysaccharide (LPS), ghrelin and oleuropein on histological findings in renal tissue of the experimental groups. LPS (*left figure*): In this field nearly all the cells lining the tubules are pale and swollen due to hydropic changes (*arrows*). *Scale bar* = 150 μm. LPS (*right figure*): glomerulonephritis; there is not cellular proliferation in the glomerulus and not thickening of basement membrane. Moreover, the walls of the capillaries are not thickened and making several loops (*arrows*). *Scale bar* = 40 μm. Ghr_1+LPS: High population of tubular cells showing hydropic degeneration (*arrows*). Ghr_4+LPS: all tubules and glomerular tufts appear to be normal. Ole_{10}+LPS: swelling tubular cells were confined in subcapsular region (*arrows*). Ole_{15}+LPS: tubular degeneration was concentrated in subcapsular area (*arrows*). Ghr_1+LPS; ghrelin 1 nmol/rat plus lipopolysaccharide, Ghr_4+LPS; ghrelin 4 nmol/rat plus lipopolysaccharide, Ole_{10}+LPS; oleuropein 10 mg/kg plus lipopolysaccharide, Ole_{15}+LPS; oleuropein 15 mg/kg plus lipopolysaccharide. All of the groups received LPS (once; 5 mg/kg) as intraperitoneally. Hematoxylin and eosin staining; S*cale bars* for ghrelin and oleuropein pretreated rats are 150 μm

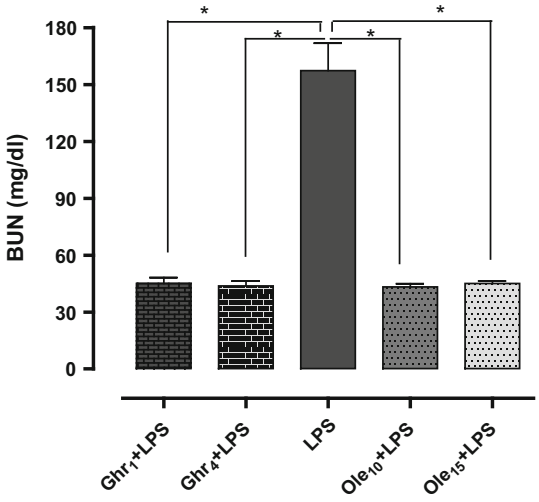

Fig. 2 The effects of lipopolysaccharide (LPS), ghrelin and oleuropein on blood urea nitrogen concentration (BUN mg/dl of serum) in the experimental groups. *Asterisk* indicates statistical difference between LPS and the other groups ($P < 0.001$). Ghr_1+LPS; ghrelin 1 nmol/rat plus lipopolysaccharide, Ghr_4+LPS; ghrelin 4 nmol/rat plus lipopolysaccharide, Ole_{10}+LPS; oleuropein 10 mg/kg plus lipopolysaccharide, Ole_{15}+LPS; oleuropein 15 mg/kg plus lipopolysaccharide. All of the groups received LPS (once; 5 mg/kg) as intraperitoneally. Data are expressed as mean ± SEM. n = 8 rats per group

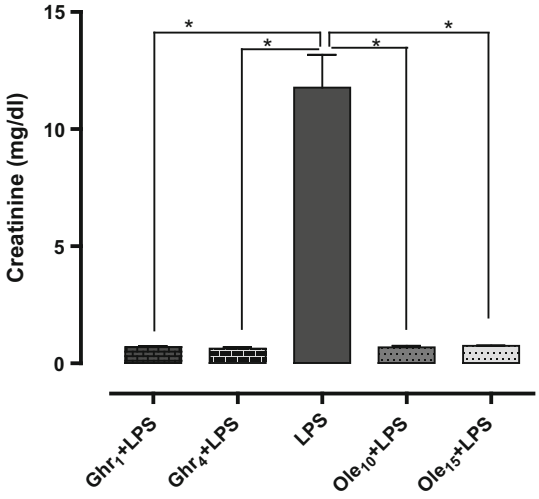

Fig. 3 The effects of lipopolysaccharide (LPS), ghrelin and oleuropein on creatinine concentration (creatinine mg/dl of serum) in the experimental groups. *Asterisk* indicates statistical difference between LPS and the other groups ($P < 0.001$). Ghr_1+LPS; ghrelin 1 nmol/rat plus lipopolysaccharide, Ghr_4+LPS; ghrelin 4 nmol/rat plus lipopolysaccharide, Ole_{10}+LPS; oleuropein 10 mg/kg plus lipopolysaccharide, Ole_{15}+LPS; oleuropein 15 mg/kg plus lipopolysaccharide. All of the groups received LPS (once; 5 mg/kg) as intraperitoneally. Data are expressed as mean ± SEM. n = 8 rats per group

et al. 2009; Tesauro et al. 2009). It is well known that physiologic activity of ghrelin is mediated by an interaction between ghrelin and GHSR (Takeda et al. 2006; Neamati

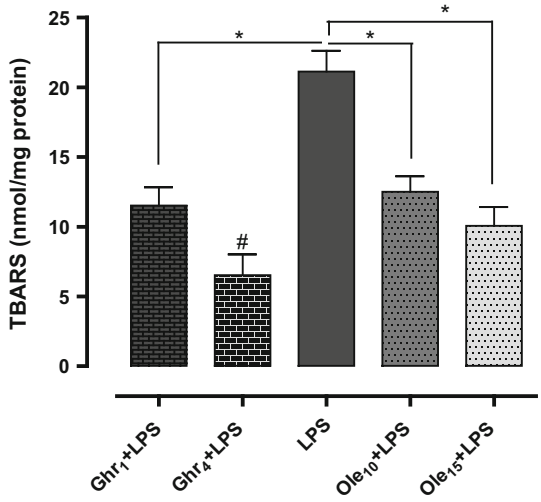

Fig. 4 The effects of lipopolysaccharide (LPS), ghrelin and oleuropein on thiobarbituric acid reactive substances concentration (nmol/mg protein of kidney tissue) in the experimental groups. *Asterisk* indicates statistical difference between LPS and the pretreated groups ($P < 0.05$). #Demonstrates statistical difference between Ghr_4+LPS group and Ole_{10}+LPS-treated rats ($P < 0.05$). Ghr_1+LPS; ghrelin 1 nmol/rat plus lipopolysaccharide, Ghr_4+LPS; ghrelin 4 nmol/rat plus lipopolysaccharide, Ole_{10}+LPS; oleuropein 10 mg/kg plus lipopolysaccharide, Ole_{15}+LPS; oleuropein 15 mg/kg plus lipopolysaccharide. All of the groups received LPS (once; 5 mg/kg) as intraperitoneally. Data are expressed as mean ± SEM. n = 8 rats per group

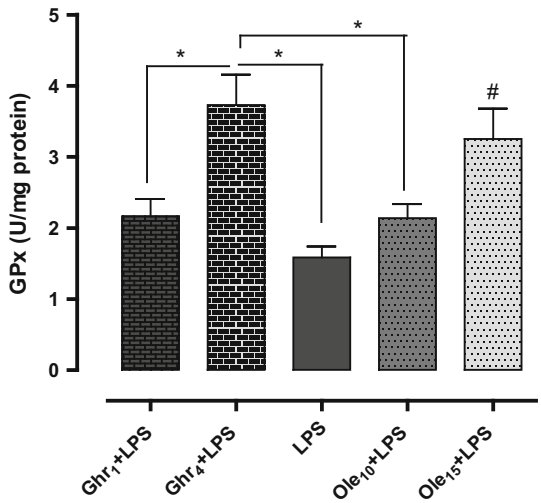

Fig. 5 The effects of lipopolysaccharide (LPS), ghrelin and oleuropein on glutathione peroxidase activity (GPx unit/mg protein of kidney tissue) in the experimental groups. *Asterisk* indicates statistical difference between Ghr_4+LPS with Ghr_1+LPS, LPS, and Ole_{10}+LPS groups ($P < 0.05$). #Demonstrates statistical difference between Ole_{15}+LPS group and LPS group ($P < 0.05$). Ghr_1+LPS; ghrelin 1 nmol/rat plus lipopolysaccharide, Ghr_4+LPS; ghrelin 4 nmol/rat plus lipopolysaccharide, Ole_{10}+LPS; oleuropein 10 mg/kg plus lipopolysaccharide, Ole_{15}+LPS; oleuropein 15 mg/kg plus lipopolysaccharide. All of the groups received LPS (once; 5 mg/kg) as intraperitoneally. Data are expressed as mean ± SEM. n = 8 rats per group

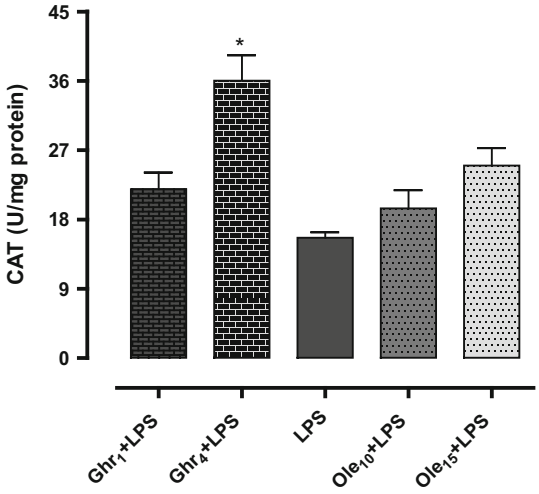

Fig. 6 The effects of lipopolysaccharide (LPS), ghrelin and oleuropein on catalase activity (CAT unit/mg protein of kidney tissue) in the experimental groups. *Asterisk* indicates statistical difference among Ghr_4+LPS and the other groups ($P < 0.05$). Ghr_1+LPS; ghrelin 1 nmol/rat plus lipopolysaccharide, Ghr_4+LPS; ghrelin 4 nmol/rat plus lipopolysaccharide, Ole_{10}+LPS; oleuropein 10 mg/kg plus lipopolysaccharide, Ole_{15}+LPS; oleuropein 15 mg/kg plus lipopolysaccharide. All of the groups received LPS (once; 5 mg/kg) as intraperitoneally. Data are expressed as mean ± SEM. n = 8 rats per group

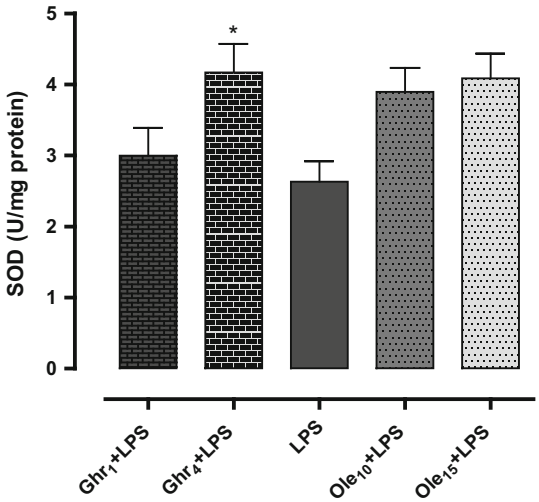

Fig. 7 The effects of lipopolysaccharide (LPS), ghrelin and oleuropein on superoxide dismutase activity (SOD unit/mg protein of kidney tissue) in the experimental groups. *Asterisk* indicates statistical difference between Ghr_4+LPS group and LPS group ($P < 0.05$). Ghr_1+LPS; ghrelin 1 nmol/rat plus lipopolysaccharide, Ghr_4+LPS; ghrelin 4 nmol/rat plus lipopolysaccharide, Ole_{10}+LPS; oleuropein 10 mg/kg plus lipopolysaccharide, Ole_{15}+LPS; oleuropein 15 mg/kg plus lipopolysaccharide. All of the groups received LPS (once; 5 mg/kg) as intraperitoneally. Data are expressed as mean ± SEM. n = 8 rats per group

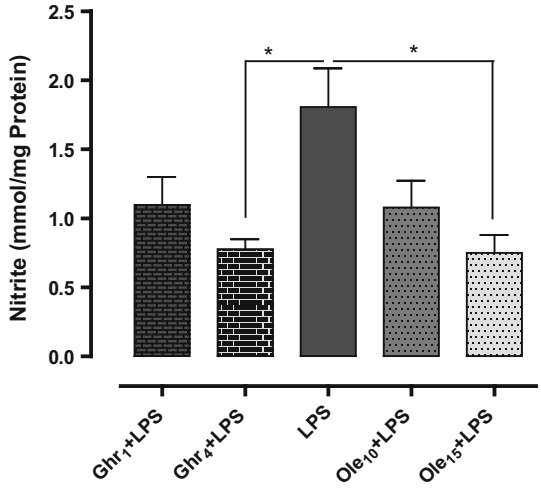

Fig. 8 The effects of lipopolysaccharide (LPS), ghrelin and oleuropein on nitrite content (Nitrite content millimol/mg protein of kidney tissue) in the experimental groups. *Asterisk* indicates statistical difference between LPS-treated group with Ghr_4+LPS and Ole_{15}+LPS groups ($P < 0.05$). There was no significant difference among LPS-treated rats with Ghr_1+LPS and Ole_{10}+LPS groups ($P > 0.05$). Ghr_1+LPS; ghrelin 1 nmol/rat plus lipopolysaccharide, Ghr_4+LPS; ghrelin 4 nmol/rat plus lipopolysaccharide, Ole_{10}+LPS; oleuropein 10 mg/kg plus lipopolysaccharide, Ole_{15}+LPS; oleuropein 15 mg/kg plus lipopolysaccharide. All of the groups received LPS (nce; 5 mg/kg) as intraperitoneally. Data are expressed as mean ± SEM. n = 8 rats per group

et al. 2011). Several studies reported that GHSR present in the pituitary, myocardium, aorta, and kidney and that various tissues, including the kidney expressed the ghrelin gene (Gnanapavan et al. 2002; Takeda et al. 2006), and upregulation of renal GHSR has also been reported during sepsis in mice (Wang et al. 2009). In this regard, ghrelin was shown to protect the kidney from LPS-mediated injury in mice (Wang et al. 2009). Although the most beneficial dosage of ghrelin is still unclear, in this study we injected 1 and 4 nmol/rat of ghrelin to examine whether these therapeutic regimens for 10 consecutive days are capable to increase renal antioxidant defense system. These protocols were based on the ghrelin concentration in the fasting state of rats, (Fernandez-Fernandez et al. 2005) and close to the dose which was used in our previous report for evaluation of antioxidant enzymes in the rat kidney (Neamati et al. 2011). We demonstrated that only 4 nmol ghrelin/rat schedule is suitable to protect renal function from oxidative and nitrosative stresses during sepsis. Despite the disordered endocrine system demonstrated in patients with renal failure, this study demonstrates that the antioxidant effects of exogenous ghrelin administration is retained in pretreated rats in particular for Ghr_4 plus LPS group. Indeed, we demonstrated a greater increase in antioxidant status of subcutaneous injection of 4 nmol ghrelin/rat than to 1 nmol/rat. This may reflect the high circulating total ghrelin levels achieved and subsequent prevention of oxidative and nitrosative stresses in Ghr_4+LPS group. Therefore, the administration of ghrelin to patients with

renal failure may yield clinical benefits beyond its anti-cataolic and antioxidant effects.

In the present study, we treated animals with two doses of oleuropein (10 and 15 mg/kg as orally) based on our previous reports (Alirezaei et al. 2011, 2012a, b; 2014). There was a significant difference in histopathological results, renal function tests, activities of antioxidant enzymes and nitrite content in rats pretreated by oleuropein (In particular 15 mg/kg schedule) versus LPS group. Oleuropein is a phenolic compound which has been shown to possess diverse healing properties for its vasodilatory, hypotensive, anti-inflammatory, anti-rheumatic, diuretic, anti-atherogenic, antipyretic and antioxidant effects (Visioli et al. 1998, 2000; Khayyal et al. 2002; Al-Azzawie and Alhamdani 2006; Alirezaei et al. 2012b). It seems that many of these pharmacologic features of oleuropein are due to its potent antioxidant actions (Manna et al. 2002, 2004). In the present study, oleuropein exerted as a natural suppressor of oxidative and nitrosative stresses which resulting in renal function tests. Oleuropein is able to chelate metal ions, such as Cu^{2+} and Fe^{2+}, which catalyze free radical generation reactions (Andrikopoulos et al. 2002). Oleuropein and its metabolite hydroxytyrosol both possess the structural requirement (a catechol group) needed for optimum antioxidant and scavenging activity (Al-Azzawie and Alhamdani 2006). Therefore, it is possible that oleuropein decreased LPS-induced oxidative stress and lipid peroxidation in this study by two pathways; First, by rapid conversion of H_2O_2 to H_2O and preventing of H_2O_2 accumulation and second, by quenching the hydroxyl radicals that, trapping of hydroxyl radicals leads to oxidative breakdown of the phenolic compounds (Alirezaei et al. 2012b). Our results are in agreement with the previous reports in liver, intestine and testes of rats (Alirezaei et al. 2011, 2012b, 2014). In respect to these results, the use of oleuropein may be of prophylactic value in reducing the complications usually resulting from oxidative stress in septic shock.

LPS induces lipid peroxidation and hydroperoxide formation, and this effect can be prevented by the addition of antioxidants (Ben-Shaul et al. 2001; Kim et al. 2004). Lipid peroxidation is a crucial factor in the propagation of cellular damage from LPS-induced oxidative stress, leading to the increased permeability of the plasma membrane (Paller et al. 1984; Noiri et al. 1996) as well as of mitochondrial membranes (Kako et al. 1988; Noiri et al. 1996). Herein, treatment with LPS alone increased TBARS concentration (as a lipid peroxidation marker), and treatment with ghrelin plus LPS decreased TBARS level when compared to LPS group. Moreover, oleuropein at dose of 15 mg/kg significantly reduced LPS-induced lipid peroxidation versus LPS group. LPS also induces nitrosative stress by elevation of NO production and peroxynitrite formation (Noiri et al. 1996). The reactivity of peroxynitrite is reported as pH dependent (Crow et al. 1994; Noiri et al. 1996). It is readily isomerized from stable *cis* to reactive *trans* peroxynitrous acid in acidic conditions like the reperfusion phase in renal failure. Among oxidative reactions of peroxynitrite, its hydroxyl radical-like reactivity is extremely potent (Beckman et al. 1990) and leads to the propagated lipid peroxidation. Numerous studies suggest that NO is capable of making alterations in the chemical biology of protein function via reaction with the cysteine residue of target proteins, a process known as *S*-nitrosylation (Amiraslani et al. 2012). It has been demonstrated that endotoxin-induced renal injury is accompanied by nitrotyrosine formation (Noiri et al. 1996, 2001; Ben-Shaul et al. 2001),"a part from inhibiting the functions of several highly susceptible enzymes, i.e., prostacyclin synthase, prostaglandin endoperoxide synthase, and Mn-SOD" (Noiri et al. 1996). SOD, an antiperoxidative enzyme in cells rapidly converts superoxide anion (O^{2-}) to less dangerous hydrogen peroxide (H_2O_2) then GPx and CAT, two key antioxidant enzymes, can decompose H_2O_2 to water (Kheradmand et al. 2010). Although, H_2O_2 is not a particularly reactive product, it can be reduced to the highly reactive metabolites hydroxyl radicals (Peltola et al. 1992). We observed a significant decrease in activity of SOD in LPS-treated rats when compared to ghrelin-treated groups particularly in Ghr_4 plus LPS group. In this regard, previous studies showed that ghrelin is able to directly inhibit vascular superoxide production in spontaneous hypertensive rats and decreases blood pressure (Kawczynska-Drozdz et al. 2006; Iseri et al. 2008). Furthermore, in vitro studies verified that ghrelin increases the mRNA levels of SOD in fish leukocytes, implicating an antioxidant mechanism of ghrelin (Yada et al. 2006). The enhancement of SOD activity in ghrelin-pretreated rats reveals a decrease in H_2O_2 accumulation, subsequent unconsumption of GPx and CAT activities to decompose H_2O_2 to water. Hence, ghrelin pretreatment caused an overall increase in GPx and CAT activities, which may be another mechanism of action of ghrelin and further highlights its potential benefits for therapeutic use in patients with renal failure.

In the present study, sepsis and endotoxemia are associated with oxidative and nitrosative stresses, which induced acute renal failure. Ghrelin appears to be more efficient to attenuate LPS toxic effects and thereby afford protection against LPS-mediated renal damages. The protective effect of ghrelin was observed when administered at dosage 4 nmol/rat. Overall, ghrelin therapy that is able to improve energy intake, reduce oxidative and nitrosative stresses and enhance renal function would be of great value to patients with renal failure.

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Conflict of interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All rats were treated humanely and in compliance with the recommendations of Animal Care Committee for Lorestan University of Medical Sciences (Khorramabad, Iran) with Approval Number: SM 90/25.

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